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—Original Article—

Preclinical evaluation of a new cryopreservation container for a limited number of human spermatozoa

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Abstract. The aim of this study was to develop a new container for cryopreservation of a limited number of spermatozoa. To evaluate the efficacy and safety of this new container, we performed preclinical evaluations using human sperm or mouse oocytes and sperm. First, using human sperm that was frozen and then thawed, we demonstrated that the sperm recovery rate using the new container was 96.7% (58/60), which was significantly higher ($P < 0.05$) than the recovery rate of 21.2% (11/52) when using the Cryotop®. Sperm motility rates were 19.2% (10/52) using the Cryotop® and 35.0% (21/60) using the new container. Second, murine epididymal spermatozoa were divided into three groups: fresh spermatozoa, spermatozoa frozen using a straw, and spermatozoa frozen using the new container. Sperm motility, sperm membrane and DNA integrity, *in vitro* development of fertilized eggs, and offspring development after embryo transfer were assessed. The motility of freeze-thawed sperm was lower in spermatozoa that were frozen using the new container than in fresh spermatozoa or those that were frozen using a straw. After intracytoplasmic sperm injection, the survival rate was 96.7% (145/150), the 2-cell development rate was 90.3% (131/145), and the blastocyst development rate was 77.2% (112/145), when using the new container. There were no differences in the sperm membrane, DNA integrity, or in the embryo development rates to the blastocyst stage among the different frozen groups. Six offspring were derived from spermatozoa freeze-thawed in the new container, and they developed normally. Thus, the new container allows easy handling of a small number of sperms and minimizes sperm loss during cryopreservation.

Key words: Container, Cryopreservation, Human sperm, Intracytoplasmic sperm injection (ICSI), Mouse sperm

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The first report on the cryopreservation of human spermatozoa was published in 1949 by Polge *et al*. [1], and the first delivery of a baby conceived using cryopreserved human spermatozoa was reported one year later by Bunge and Sherman [2]. Since then, assisted reproduction technology, including the cryopreservation of human spermatozoa, has continuously progressed and is currently routinely used in clinical settings. Moreover, with advances in intracytoplasmic sperm injection (ICSI) methods, several pregnancies involving spermatozoa from patients with severe oligozoospermia have been reported [3, 4]. Recently, using simple-testicular sperm extraction (TESE) and micro-TESE techniques, spermatozoa were obtained from 32–62% of patients with non-obstructive azoospermia, enabling these patients to have children [5].

During assisted reproduction, spermatozoa need to be preserved until oocytes are selected and when only a limited number of spermatozoa are obtained, an appropriate cryopreservation technique is important. In conventional sperm-freezing methods, spermatozoa are dispersed in approximately 0.1–1 ml of freezing medium and then transferred to cryogenic vials or straws for freezing in liquid nitrogen. To collect preserved spermatozoa, the container (cryogenic vial or straw) is removed from liquid nitrogen and thawed in water at 37°C. The sperm suspension is then transferred to another container, mixed with washing medium, and centrifuged to obtain motile spermatozoa. If the patient has a large number of spermatozoa, then sufficient spermatozoa can be frozen and eventually retrieved, even if some spermatozoa are lost during the thawing and washing processes. In such cases, the number of spermatozoa recovered would be sufficient for ICSI. However, if this approach is used when small numbers of spermatozoa are available, then the loss of 5–10% of spermatozoa due to the large volume of medium used for suspension and the tube exchange after washing, would result in an insufficient number of retrieved spermatozoa for ICSI.

Several cryopreservation methods for small numbers of spermatozoa have been reported. One method involves the insertion of spermatozoa into an empty zona pellucida [6]. If an empty zona pellucida cannot be obtained from the patient's partner, then a zona pellucida from another species can be used [5]. When agarose gel capsules are used for cryopreservation, the embryologist must insert a glass micropipette into the agarose gel capsule for sperm preparation and then place the agarose gel capsule in the Cryotop®[7]. This procedure requires specialized skills and two devices.

Moreover, Cryotop®s are used for oocyte [8], embryo, and spermatozoon [9] preservation, even though they were not developed for sperm cryopreservation. Thus, new techniques or devices that

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Fig. 1. The new container. (A) The size of the new container was W30 × D10 × H5 mm. This size was chosen to allow the easy insertion of the container into a 1.8-ml cryotube. (B) The container (MAYU, STREX) is made of polydimethylsiloxane. The bottom of the container is transparent, which allows the assessment of sperm shape by microscopy. (C) The new container in a 1.8-ml cryotube.

allow easy freezing and thawing in a short period of time, without sperm loss, need to be developed.

In the present study, we aimed to develop a simple freezing and thawing method that can be used directly in the dish where micromanipulations are performed, for cases in which limited numbers of spermatozoa are available. This method involves the use of a new container that does not require tube exchange, and thus limits the loss of spermatozoa. The present study aimed to assess the usefulness and safety of this new container using human and murine spermatozoa.

Materials and Methods

Patients

Semen samples were obtained from patients undergoing *in vitro* fertilization at the Yamashita Shonan Yume Clinic. Ten patients were considered to have normal semen and one patient was diagnosed with oligoasthenoteratozoospermia (OAT). Written informed consent was obtained from all patients, and the study was approved by Institutional Review Board of the Yamashita Shonan Yume Clinic.

Preparation of human sperm suspensions

Semen samples were collected through masturbation from patients who maintained ejaculatory abstinence for the previous 3–5 days. Sperm parameters were assessed according to the World Health Organization criteria (2010) [10]. Sperm suspensions were prepared as previously reported [11]. In brief, seminal fluid was removed by washing the sample in a cleavage medium (SAGE cleavage medium; Cooper Surgical, Trumbull, CT, USA) supplemented with 10% plasma protein fraction (PPF; Baxter Healthcare, Deerfield, IL, USA). Samples were then centrifuged with a 2-layer Percoll density gradient, at 600 × g for 15 min and subsequently, concentrated by centrifugation at 400 × g for 5 min. Finally, the concentrated sample was resuspended in cleavage medium supplemented with 10% PPF.

Cryopreservation and thawing of human spermatozoa

Sperm suspensions were mixed with an equal volume of Sperm Freeze Solution (Vitrolife, Gothenburg, Sweden). The Sperm Freeze Solution was added dropwise to the sperm suspension and the mixture was kept at room temperature for 10 min. Fifty-two spermatozoa were aspirated using a glass micropipette (PIN07-20FT; Prime Tech, Ibaraki, Japan) and added to 1 µl of freezing medium on the edge of Cryotop® under a microscope (IX73; OLYMPUS, Tokyo, Japan; n = 10). Cryotop®s with the spermatozoa were placed in liquid nitrogen and stored. The Cryotop® was placed 4 cm above the surface of the liquid nitrogen for 2 min and then quickly immersed.

To thaw the samples, the frozen Cryotop® was removed from liquid nitrogen and placed in 2 µl of Quinn's Advantage™ cleavage medium (Cooper Surgical), supplemented with 10% PPF, at 37°C. The Cryotop® surface was carefully washed twice with 2 µl of the same medium [9]. Forty-four spermatozoa from normal patients and 16 spermatozoa from an OAT patient were aspirated using a glass micropipette and placed as a drop on the new container under a microscope (n = 11; Fig. 1; (MAYU, STREX, Osaka, Japan). The new container was then placed in a freezer maintained at −80°C for 5 min and was subsequently, inserted into a cryotube and placed in liquid nitrogen for storage. To thaw these samples, the new container was removed from liquid nitrogen and placed on a hot plate at 37°C for 2 min. The new container was then placed on a microscope stage and spermatozoa were aspirated using a glass micropipette and then added to Quinn's Advantage™ Medium with HEPES (Cooper Surgical), supplemented with 10% PPF. When these human spermatozoa were thawed, the new container was used instead of a glass micropipette and Cryotop®.

Assessment of the freezing and warming rates of the Cryotop® and new containers

We connected a thermoelectric couple (KT-0177C4436; CHINO, Tokyo, Japan) to a digital MC series thermometer (CHINO) and fixed the edge of the thermoelectric couple to the Cryotop® or to the inner part of the new container (Fig. 1). We then measured the temperature at 1 minute intervals. The Cryotop® was placed 4 cm above the surface of the liquid nitrogen for 2 min and was then quickly immersed [9]. It was then removed and placed in cleavage medium at 37°C and the time required to reach 37°C was determined.

In contrast, the new container was first placed in a freezer (Program Deep Freezer; STREX) at −80°C for 5 min and then placed in liquid nitrogen. The time required for the container to reach −196°C from −80°C was determined. The new container was then removed from liquid nitrogen and placed on a hot plate at 37°C, and the time required to reach 37°C was determined.

Animals

BDF1 and ICR mice (8–12 weeks old) were purchased from CLEA Japan (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions. They were provided with water and commercial laboratory mouse chow *ad libitum* and were housed under controlled lighting conditions (light: 0800–2000 h). All experiments were reviewed and approved by the Animal Experimental Committee at Toin University.

Collection of murine oocytes

Female BDF1 mice were intraperitoneally injected with 7.5 units of equine chorionic gonadotropin (Aska Pharmaceuticals, Tokyo, Japan) and 48–50 h later, they were injected with 7.5 units of human chorionic gonadotropin (hCG; Aska Pharmaceuticals). Mature oocytes were collected from the oviducts, 14–15 h after hCG injection and were freed from cumulus cells by pipetting after a 3-min treatment with hyaluronidase (ART-4007-A; Cooper Surgical) in M16 medium (M7292; Sigma Aldrich, St Louis, MO, USA). Oocytes were cultured in M16 medium at 37°C under 5% CO_2 until they were used for microinjections.

Cryopreservation and thawing of murine spermatozoa

Spermatozoa obtained from 8-week-old male BDF1 mice were frozen using the new container (n = 10) or straws (n = 10). For comparison with frozen spermatozoa, fresh spermatozoa were collected from male BDF1 mice (n = 10) and were not frozen. The fresh spermatozoa were incubated in M16 medium at 37°C under 5% CO_2 until further use.

Spermatozoa were dispersed in 120 µl of sperm-freezing medium (FERTIUP®; KYUDO, Saga, Japan) [12, 13] and were maintained at 37°C for 3 min. Half of the culture was transferred to 10-µl straws (MY SCIENCES, Tokyo, Japan). These straws were placed in liquid nitrogen vapor for 5 min and then stored in liquid nitrogen [14]. By contrast, 10 µl of the sperm suspension was placed at the bottom of the new container and covered with 200 µl of mineral oil (93621, Mineral Oil Heavy, Kitazato Corp., Shizuoka, Japan). We used a programable deep freezer (STREX) maintained at −80°C. The new container was frozen at −80°C for 5 min, transferred to a cryotube (Sumitomo Bakelite, Tokyo, Japan), and stored in liquid nitrogen until use. The frozen straws were warmed in water at 37°C for 10 min and 100 µl of M16 medium was added. The frozen new containers were warmed on a hot plate at 37°C for 2 min and 10 µl of the thawed sperm suspension was aspirated and dispersed in 50 µl of M16 medium. Ten minutes after warming, sperm motility was compared between spermatozoa frozen using the straw (n = 10) and those frozen using the container (n = 10), via visual inspection using a Makler chamber. To assess sperm motility, 10 µl of thawed sperm suspension was placed on a Makler chamber and covered with a cover glass. The number of spermatozoa was counted in more than 5 fields and the average of each field was calculated. The average number of spermatozoa was $47.8 \pm 5.3 \times 10^6$/ml in fresh samples, $44.1 \pm 12.3 \times 10^6$/ml in straws, and $28.8 \pm 4.6 \times 10^6$/ml in the new container. Spermatozoa with forward motility, with the exception of non-progressive motile spermatozoa, were considered motile.

When murine spermatozoa were thawed, we inserted a straw directly into the M16 medium. However, we used a pipette to pick up murine spermatozoa from the new container.

Assessment of sperm membrane integrity

Staining was performed on fresh spermatozoa (n = 5) and on thawed spermatozoa from the new container (n = 5) and from straws (n = 5). Spermatozoa were stained with Hoechst 33342 (H1399; Life Technologies, Gaithersburg, MD, USA) and propidium iodide (PI, P3566, Life Technologies) according to the method described by Diercks A-K *et al*. [15]. To 200 µl of M16 medium, 0.001 mg/ml of Hoechst 33342 and 0.0001 mg/ml of PI were added. The sperm suspensions from each group were then mixed in the M16 medium containing the two dyes and were maintained at 37.5°C for 10 min in 1.5-ml microtubes. Thereafter, samples from each suspension were dropped on glass slides (S9441; Matsunami Glass, Osaka, Japan) and covered with cover glasses. Five glass slides were prepared for each group and were microscopically analyzed using an Olympus fluorescence microscope, with FUW and FGW filters. A minimum of 100 spermatozoa were evaluated on each slide.

Assessment of sperm DNA integrity

Chromomycin A3 (CMA3, C2659, Sigma Aldrich) competes with protamines for binding to the minor groove of DNA. CMA3 is a simple and useful tool for assessing the packaging of sperm chromatin and it allows indirect visualization of protamine deficiency. The spermatozoa remaining after microinjection were fixed with Carnoy's solution (methanol: glacial acetic acid 3:1) by incubation at 4°C for 5 min. Thereafter, the suspension was smeared on glass slides (S9441, Matsunami Glass) and smears were treated for 20 min with 200 µl of CMA3 solution (0.25 mg/ml CMA3 in Mcllvaline buffer [040-33731; Wako Pure Chemical Industries, Osaka, Japan]). The slides were then washed in PBS and analyzed under a fluorescence microscope (IX73; OLYMPUS, Tokyo, Japan) with an FVW filter. A minimum of 100 spermatozoa were evaluated on each slide and were counted on the basis of cell brightness [16].

Microinjection for oocytes

Intracytoplasmic injection of freeze-thawed spermatozoa were performed using a glass micropipette attached to a piezo-electric actuator (Prime Tech), as previously described [17]. The lid of a 6-well plastic dish (Research Institute for the Functional Peptides, Yamagata, Japan) was used as a microinjection chamber. A few 5-µl drops of M2 medium and a 10% polyvinylpyrrolidone (PVP) solution with human serum albumin (5 mg/ml; 90123; Irvine Scientific, Santa Ana, CA, USA) were placed at the bottom of the chamber and covered with mineral oil. Freeze-thawed spermatozoa were then placed in PVP. Single, motile spermatozoa were caught in an injection pipette (PINU06-20FT, Prime Tech), and the head was separated from the tail by applying several piezo pulses. Each isolated sperm head was injected into an oocyte in M2 medium, under an inverted microscope (TE2000, NIKON, Tokyo, Japan). The injected oocytes were then kept in M2 medium for 10 min and were then cultured in M16 medium at 37°C under 5% CO_2 for 120 h.

Embryo transfer

Embryos that reached the blastocyst stage after 120 h of culture in M16 medium were transferred to the uteri of pseudo pregnant

female ICR mice (5–10 embryos per recipient), after mating with vasectomized male ICR mice (day 3.5). Generally, 72-h embryos are transferred at 2.5 d.p.c. However, in this study, 120-h embryos were transferred at 3.5 d.p.c to obtain data regarding the *in vitro* development to the blastocyst stage. On day 19.5, recipient mice were killed by cervical dislocation and their uteri were examined for the presence of live, term offspring. Foster mother mice were prepared to raise the offspring. The growth of the offspring was carefully monitored.

Statistical analysis

Each experiment was repeated at least three times. Data were analyzed by *t*-test or Chi-squared test. Average rate data were transformed using arcsine transformation prior to analysis.

Results

Comparison of freezing and warming rates between the Cryotop® and new containers

The freezing and warming rates of the Cryotop® and the new container are presented in Fig. 2. The Cryotop® was placed in liquid nitrogen vapor for 2 min and the cooling rate was −25.1°C/sec down to −80°C. It was then plunged into liquid nitrogen, after which the cooling rate was −100.8°C/sec down to −180°C.

By contrast, the new container was first placed in a freezer (−80°C) for 5 min, where the cooling rate was −9.0°C/sec down to −83.5°C. It was then plunged into liquid nitrogen, after which the cooling rate was −49.3°C/sec down to −180°C. In thawing tests, the Cryotop® took 15 seconds to increase from −196°C to room temperature, whereas the new container took 200 seconds to increase from −196°C to 37°C.

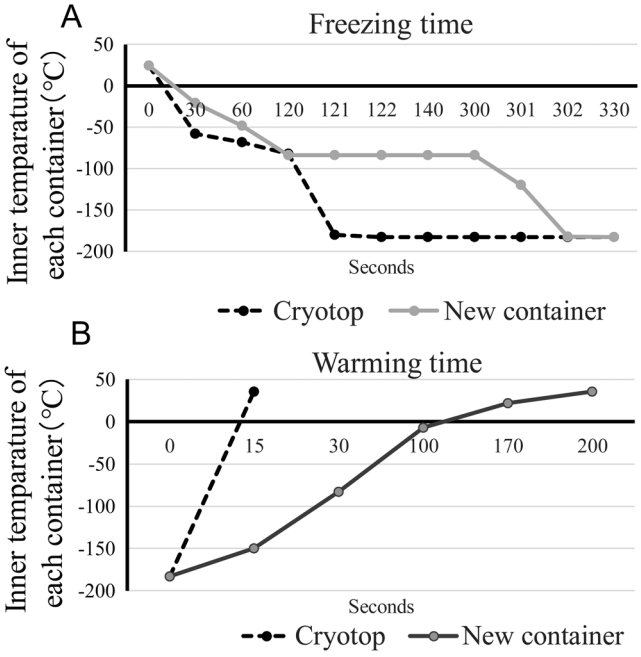

Fig. 2. Comparison of freezing and warming rates between the Cryotop® and the new container. (A) The freezing rates of the Cryotop® and the new container. The Cryotop® was placed 4 cm above the surface of the liquid nitrogen for 2 min and was then quickly immersed. The new container was first placed in a freezer at −80°C for 5 min and then placed in liquid nitrogen. (B) The warming rates of the Cryotop® and the new container. The Cryotop® was removed from liquid nitrogen and placed in cleavage medium at 37°C. The new container was removed from liquid nitrogen and placed on a hot plate at 37°C.

Comparison of the recovery and motility of freeze-thawed human spermatozoa between the Cryotop® and new containers

The recovery and motility rates of freeze-thawed human spermatozoa obtained from 10 patients were compared between the Cryotop® and the new container (Table 1). The mean motility rate before freezing was 79.5 ± 12.1%. The rates of sperm recovery after

Table 1. Comparison of recovery efficiency of freeze-thawed normal human and OAT patient's spermatozoa using the Cryotop® and new containers

Patient no.	Sperm density *	Motility rate of fresh spermatozoa **	Cryotop®			New container		
			No. of frozen spermatozoa	No. of detected spermatozoa (%)	No. of motile spermatozoa (%)	No. of frozen spermatozoa	No. of detected spermatozoa (%)	No. of motile spermatozoa (%)
1	167	130 (78)	5	3 (60)	2 (66.7)	5	5 (100)	2 (20)
2	73	56 (77)	5	1 (20)	1 (100)	4	4 (100)	2 (20)
3	118	89 (75)	5	0 (0)	0 (0)	4	4 (100)	2 (50)
4	80	71 (89)	5	0 (0)	0 (0)	5	5 (100)	2 (20)
5	251	203 (81)	5	0 (0)	0 (0)	4	4 (100)	1 (25)
6	171	151 (88)	5	2 (20)	2 (100)	3	3 (100)	2 (66.7)
7	25.4	12.0 (47)	5	0 (0)	0 (0)	5	5 (100)	1 (20)
8	19.6	17.9 (91)	5	1 (20)	1 (100)	5	5 (100)	1 (20)
9	173	150 (87)	5	3 (60)	3 (100)	4	4 (100)	2 (50)
10	520	390 (75)	7	1 (14.3)	1 (100)	5	5 (100)	2 (20)
11	–	–	–	–	–	16	14 (87.5)	4 (28.6)
	***	****	52	11 (21.2) [a]	10 (19.2)	60	58 (96.7) [b]	21 (35.0)

[a–b] $P < 0.05$ between values with different superscript letters. * Sperm density × 10^6/ml. ** Motility rate of fresh spermatozoa × 10^6/ml (%). *** Average sperm density ± SE (Nos.1–10): 159.8 ± 138.5. **** Average motility rate of fresh spermatozoa ± SE (Nos.1–10): 126 ± 105.3/79.5 ± 12.

thawing were 21.2% (11/52) with the Cryotop® and 96.7% (58/60) with the new container ($P < 0.05$). Additionally, the motility of spermatozoa after thawing was 19.2% (10/52) with the Cryotop® and 35.0% (21/60) with the new container. The rates of sperm recovery and motility with the new container were also assessed in a patient with OAT. Sixteen spermatozoa were frozen in the new container. After thawing, 14 spermatozoa were recovered (87.5%) and four (28.6%) of them were found to be motile (Table 1).

Motility of freeze-thawed murine spermatozoa

The motilities of spermatozoa freeze-thawed in the straw and those freeze-thawed in the new container were significantly lower than the of motility of fresh spermatozoa ($P < 0.001$ for both). Additionally, the motility of spermatozoa freeze-thawed in the new container was significantly lower than the motility of those freeze-thawed in the straw ($P < 0.001$, Table 2).

Membrane integrity of fresh and freeze-thawed murine spermatozoa

The typical appearances of Hoechst 33342 and PI-stained murine spermatozoa are shown in Fig. 3A. Using bright-field microscopy, we counted 935 spermatozoa in fresh samples, 1,073 spermatozoa in the straw, and 960 spermatozoa in the new container. The rates of positive Hoechst 33342 staining were 99.8% (933/935) for fresh spermatozoa, 99.3% (1,066/1,073) for spermatozoa freeze-thawed in the straw, and 99.1% (951/960) for those freeze-thawed in the new container. There were no differences in the rates of positive Hoechst 33342 staining between groups. The positive rates of PI staining were significantly lower in fresh spermatozoa (23.2 ± 20.7%) than in spermatozoa freeze-thawed in the straw (62.6 ± 26.4%) and those freeze-thawed in the new container (52.8 ± 31.0%, both $P < 0.001$, Fig. 3B). However, the rate of PI staining did not differ between spermatozoa freeze-thawed in the straw and those freeze-thawed in the new container ($P = 0.76$).

DNA integrity of freeze-thawed murine spermatozoa

Figure 4 shows a typical result of CMA3 staining of murine spermatozoa. There were no differences in CMA3 staining among fresh spermatozoa and those freeze-thawed in the straw or the new container. The rate of positive CMA3 staining in fresh spermatozoa (2.4 ± 2.1%) was not significantly different from the rate of positive CMA3 staining after freeze-thawing in the straw (3.3 ± 5.6%, $P = 0.951$). When we used the new container, the rate of positive CMA3 staining after thawing was 2.4 ± 6.4%, which was not significantly different from the straw ($P = 0.33$) or fresh ($P = 0.461$) spermatozoa.

In vitro and in vivo development of embryos derived from freeze-thawed murine spermatozoa

The fertilization and embryo development rates after the injection of oocytes with freeze-thawed spermatozoa (straw or new container) or fresh spermatozoa are presented in Table 3. After ICSI, more than 90% of oocytes survived using a piezo micromanipulator in all three groups. The cleavage rates were 100% (99/99) for fresh spermatozoa, 86.6% (110/127) for spermatozoa freeze-thawed in the straw, and 90.3% (131/145) for those freeze-thawed in the new container. The cleavage rate observed with the new container was not significantly different from the rate with the straw ($P = 0.06$), but was lower than the rate for fresh spermatozoa ($P < 0.05$). Moreover, blastocyst development rates were 91.9% (91/99) for fresh spermatozoa, 80.3% (102/127) for spermatozoa freeze-thawed in the straw, and 77.2% (112/145) for those freeze-thawed in the new container. The blastocyst development rate observed with the new container was not significantly different from the rate with the straw ($P = 0.22$), but was lower than the rate for fresh spermatozoa ($P < 0.05$).

Table 2. Motility rates of fresh and freeze-thawed murine spermatozoa

Spermatozoa conditions	N	Forward motility after thawing ± SE (%)
Fresh	3	74.9 ± 7.2 [a]
Straw	3	20.4 ± 2.7 [b]
New container	3	6.8 ± 2 [c]

[a, b, c] $P < 0.001$ between values with different superscript letters.

The results of embryo transfer into the uteri of recipient mice are shown in Table 3. Implantation rates were 57.4% (27/56) for fresh spermatozoa, 23.7% (18/91) for spermatozoa freeze-thawed in the straw, and 29.2% (19/78) for those freeze-thawed in the new container. Implantation rates were significantly lower for fresh spermatozoa than for those freeze-thawed in the straw or the new container (both $P < 0.01$). However, the implantation rate did not differ between spermatozoa freeze-thawed in the straw and those freeze-thawed in the new container ($P = 0.28$).

The embryos derived from spermatozoa frozen-thawed in the new container showed normal morphology similar to that of embryos derived from the other groups. The offspring and placental weights of the three groups are presented in Table 3.

The weight of offspring derived from spermatozoa freeze-thawed in the straw, those freeze-thawed in the new container, and fresh spermatozoa were 1.56 ± 0.17 g (n = 8), 1.39 ± 0.28 g (n = 6), and 1.28 ± 0.25 g (n = 6), respectively. Offspring derived from spermatozoa freeze-thawed in the straw weighed significantly more than those freeze-thawed in the new container or fresh spermatozoa (both $P < 0.05$). The weight of placentas derived from spermatozoa freeze-thawed in the straw, those freeze-thawed in the new container, and fresh spermatozoa were 0.19 ± 0.02 g (n = 8), 0.15 ± 0.02 g (n = 6), and 0.15 ± 0.03 g (n = 6), respectively. The placentas derived from spermatozoa freeze-thawed in the straw weighed significantly more than those freeze-thawed in the new container or fresh spermatozoa (both $P < 0.05$). The six offspring derived from spermatozoa freeze-thawed in the new container were successfully weaned and demonstrated normal growth.

Discussion

In this study, a new cryopreservation container was used, and its safety was investigated. The major advantages of this container over previously used devices are as follows: 1) the sheet of the container allows clear visual field observation, as with a plastic dish; 2) the material is stretchable, and thus, it will not break even if placed in liquid nitrogen; 3) the container is designed to fit into a cryotube for long-term storage in liquid nitrogen and thus, no other special storage containers are required; 4) the material has good heat conduction

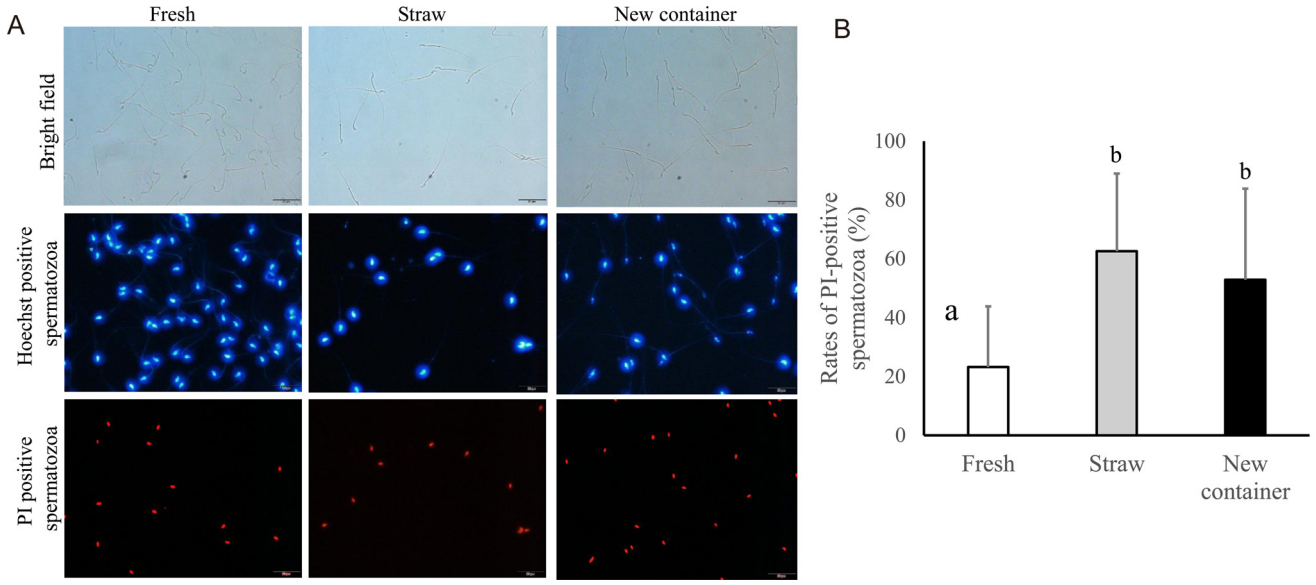

Fig. 3. Hoechst 33342 and propidium iodide (PI) staining of fresh and freeze-thawed murine spermatozoa. (A) Hoechst 33342-positive murine spermatozoa appear bright blue, whereas PI-positive spermatozoa appear bright red. Bar: 20 µm. Images of spermatozoa freeze-thawed in a straw, those freeze-thawed in the new container, and fresh spermatozoa are presented. Staining was repeated five times for each group (n = 5). Blue indicates sperm DNA and red indicates dead spermatozoa. Spermatozoa with heads that stained only blue were considered viable. (B) Rates of PI-positive spermatozoa freeze-thawed in straw, those freeze–thawed in the new container, and fresh spermatozoa. The rates were significantly lower for fresh spermatozoa than for freeze-thawed spermatozoa in the straw and the new container (both $P < 0.001$).

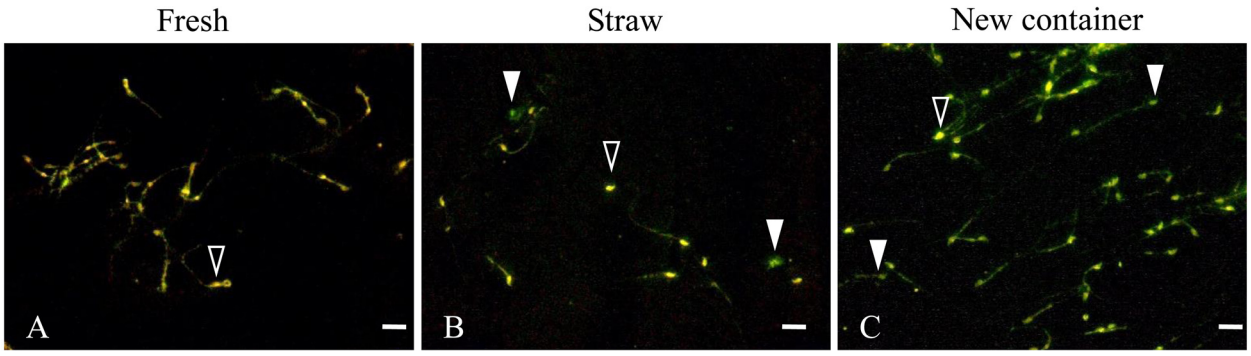

Fig. 4. CMA3 staining of fresh or freeze-thawed murine spermatozoa. CMA3-positive murine spermatozoa appear bright green, while CMA3-negative spermatozoa appear bright yellow. Bar: 20 µm. Picture of fresh spermatozoa (A) and spermatozoa in the straw (B) and the new container (C). Staining was repeated three times in the three groups (n = 3). The yellow color indicates non-damaged sperm and the green color indicates sperm with damaged nuclei. Spermatozoa with green heads were defined as CMA3-positive (Δ) and those with yellow heads were defined as CMA3-negative (▲).

and allows for the thawing of frozen spermatozoa within 2 min; and 5) freeze-thawed spermatozoa can be washed via aspiration with a glass micropipette and transferred to a minimal culture medium (not containing a frozen solution), thereby avoiding centrifugation. Thus, the preparation processes are simplified and sperm loss is substantially minimized prior to microinjection. This container also allows the cryopreservation of small numbers of spermatozoa without the requirement of specialized skills.

We aimed to assess the utility of this new container for processing and storing human sperm samples. We compared the Cryotop® with the new container for sperm recovery and motility rates after thawing. We did not observe any improvements in sperm recovery or motility compared with the results obtained by Endo *et al*. [9].

Before freezing sperm samples, we inserted 5 spermatozoa into freezing medium in Cryotop®s using a glass micropipette. However, we were not able to observe the spermatozoa due to the shadow of the freezing medium. After thawing, a very small volume of freezing medium was left in the Cryotop®. Sperm was found in the medium, but it was not able to be collected because we didn't use a glass micropipette, but instead, inserted the Cryotop® into the flat

Table 3. *In vitro* and *in vivo* development of murine embryos derived from oocytes injected with fresh or freeze-thawed murine spermatozoa

	Preservation conditions		
	Fresh	Straw	New container
No. of oocytes	110	137	150
No. (%) of oocytes surviving after ICSI	99 (90.0)	127 (92.7)	145 (96.7)
No. (%) of two-cell embryos	99 (100) [a]	110 (86.6) [b]	131 (90.3) [b]
No. (%) of blastocysts	91 (91.9) [c]	102 (80.3) [d]	112 (77.2) [d]
No. of transferred blastocysts	56	91	78
No. of recipients	5	8	7
No. of implanted embryos (per transferred, %)	27 (57.4) [e]	18 (23.7) [f]	19 (29.2) [f]
No. of offspring (per transferred, %)	14 (29.8)	13 (17.1)	11 (16.9)
No. of offspring measured for body and placenta weight *	6	8	6
Average of offspring weight (g)	1.28 ± 0.25 [g]	1.56 ± 0.17 [h]	1.39 ± 0.28 [g]
Average of placenta weight (g)	0.15 ± 0.03 [i]	0.19 ± 0.02 [j]	0.15 ± 0.02 [i]

[a–b, c–d] $P < 0.05$ between values with different superscript letters. [e–f, g–h, i–j] $P < 0.01$ between values with different superscript letters. * Part of the recipient mice delivered naturally. However, naturally delivered offspring were killed by recipient mice. We were able to measure the body and placenta weight of surviving offspring born by caesarian section. Therefore, the number of offspring that were measured for body and placenta weight was less than total number of offspring.

droplet of medium. For oocyte and embryo freezing, the Cryotop® functions well. Because oocyte and embryos are larger than sperm, they were easy to observe in the Cryotop® and were quickly released into the thawing medium. However, smaller cells such as sperm, were difficult to release into the thawing medium and therefore, it may be difficult to collect all of the sperm from a Cryotop®.

In the murine sperm study, sperm motility rates were low when spermatozoa were freeze-thawed in the straw or in the new container, compared to the motility rates of fresh spermatozoa and motility rates were lowest when using the new container. Moreover, when mouse spermatozoa were frozen, the recovery rate of sperm after thawing in the new container was half the rate of recovery of sperm samples in the straw. This difference occurred because spermatozoa in the freezing medium remained on the bottom of the new container after thawing and were not able to be collected using a pipette. When we froze a limited number of human spermatozoa using the new container, we were able to capture the spermatozoa and insert them into the freezing medium using a glass micropipette. However, when we froze a normal number of murine spermatozoa in the new container, it was very difficult to collect the adhered spermatozoa on the bottom of the container using a pipette. To increase the recovery rate when using a normal number of murine spermatozoa, the freezing method will need to be improved.

Additional freezing conditions need to be assessed to determine the optimal method for preserving murine sperm motility and achieving acceptable rates of recovery using the new container. Spermatozoa freeze-thawed in the straw and the new container showed the same degree of damage to their membranes. However, fresh spermatozoa and freeze-thawed spermatozoa had similar chromatin staining with CMA, suggesting no significant damage to their nuclei.

All oocytes showed cleavage after ICSI and there was no difference among the three groups with regard to the rate of embryonic development and embryo morphology. Thus, the cryopreservation of spermatozoa using the new container did not have a negative effect on embryo development. In the present study, 11 offspring were delivered after embryo transfer, but 5 offspring derived from spermatozoa in the new container were killed by recipient mothers after natural delivery. The killing of these 5 offspring may have been the result of very small litter sizes per recipient mouse and stress regarding cage replacement during pregnancy. Six offspring survived after caesarian section, were weaned by their foster mothers, and grew normally, indicating that sperm cryopreservation using the new container is safe.

In conclusion, this study confirmed the utility and safety of the new container. The new container allows the easy handling of a small number of spermatozoa and minimizes sperm loss during cryopreservation. Our findings will help in the development of conditions for the use of the new container in clinical settings. In future experiments, we plan to assess various cryopreservation conditions with the new container using human spermatozoa.

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